Effect of exogenous glutathione in extender on the freezability of Nili-Ravi buffalo (*Bubalus bubalis*) bull spermatozoa

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The study was designed to investigate the effect of exogenous glutathione supplementation (0.0, 0.5, 1.0, 2.0 and 3.0 mM) in *tris*-citric acid extender on post-thaw sperm motility, viability, plasma membrane and acrosomal integrity of buffalo (*Bubalus bubalis*) spermatozoa at 0, 3 and 6 hours after thawing. Glutathione supplementation of the extender upto 2.0 mM concentration increased (P≤0.05) sperm motility, viability, plasma membrane integrity and acrosomal integrity at 0, 3 and 6 hours after thawing in a dose-dependent manner compared to the control. However, glutathione supplementation at higher concentration (3.0 mM) was not beneficial for any of semen quality parameters. It is concluded that glutathione supplementation (up to 2.0 mM) of the extender improves the post-thaw quality of buffalo bull spermatozoa.

It is now well established that the process of freezing and thawing semen results into the production of reactive oxygen species (ROS). ROS are a source of stress to the spermatozoal bio-membrane system, and alteration in the antioxidant defense system [Bilodeau *et al*. 2000, Gadea *et al*. 2004] results in poor viability

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and low fertilization potential of the spermatozoa [Baily *et al*. 2000]. Freezing of the spermatozoa accelerates the ROS production and a number of studies have shown that excessive generation of ROS causes oxidative damage to the spermatozoa affecting their structure and function [Bilodeau *et al*. 2001, Chatterjee *et al*. 2001]. While the high content of polyunsaturated fatty acids in sperm plasma membrane is required to maintain unique functions at the cellular level, it makes the sperm highly susceptible to the oxidative stress, mainly because of higher lipid peroxidation levels [Kankofer *et al*. 2005].

Mammalian sperm is equipped with a defense system that comprises of both enzymatic and non-enzymatic antioxidants which offer protection against the ROS. But this indigenous defensive mechanism of the sperm is reported to be insufficient to cope with the oxidative stress [Nichi *et al*. 2006]. Moreover, antioxidant levels of semen decrease as a result of cryopreservation [Bilodeau *et al*. 2000]. However, addition of antioxidants in the extender has been reported to improve the quality of preserved mammalian semen [Bilodeau *et al*. 2001].

Glutathione is a tripeptide naturally occurring in semen and providing intracellular defense to the sperm against the oxidative stress caused by an over-production of ROS during the freezing and thawing process. Freeze-thawing of the semen causes a significant reduction in the glutathione content of the boar [Gadea *et al*. 2004] and bull semen [Bilodeau *et al*. 2000]. Moreover, supplementation of glutathione in the extender has been shown to provide a protective role in maintaining the quality of bovine [Bilodeau *et al*. 2001, Gadea *et al*. 2007] and buck semen [Sinha *et al*. 1996]. We hypothesized that exogenous glutathione supplementation of semen extender may improve post-thaw quality (motility, viability, plasma membrane integrity and normal apical ridge) of buffalo spermatozoa. Therefore, the present study was conducted to determine the effect of various glutathione concentrations in semen extender on post-thaw quality of the spermatozoa of Nili-Ravi buffaloes.

Material and methods

Preparation of extenders

Experimental extenders were prepared using *tris*-citric acid as a buffer (pH 7.0, osmotic pressure 320 mOsmol Kg^{-1}) that consisted of 1.56 g citric acid (FISHER SCIENTIFIC, UK) and 3.0 g *tris*-(hydroxymethyl)-aminomethane (RESEARCH ORGANICS, USA) dissolved in 74 ml distilled water. Fructose (SCHARLAU, Spain) 0.2% wt/vol; glycerol (RIEDEL-deHAEN, Germany) 7%; egg yolk 20% v/v; gentamycin sulphate (RECKITT BENCKISER, Pakistan) 500 µg/ml; tylosin tartrate (VMD, Belgium) 100 µg/ml; lincomycin hydrochloride (PHARMACIA & UPJOHN, Belgium) 300 µg/ml and spectinomycin hydrochloride (PHARMACIA & UPJOHN, Belgium) 600 µg/ml were added to the extender. Five experimental extenders were prepared by adding glutathione (MERCK, Germany) at the rate of 0.0, 0.5, 1.0, 2.0 or 3.0 mM in the extender.

Collection and preliminary evaluation of semen

Five adult Nili-Ravi buffalo bulls (*Bubalus bubalis*) were used for the study. Two consecutive ejaculates were collected from each bull in a graduated tube with the help of artificial vagina (42°C) at weekly intervals for a period of 3 weeks (replicates). The semen was transferred to laboratory within minutes of collection. Visual motility of the spermatozoa was assessed microscopically (at 200x) with closed circuit television. Sperm concentration was assessed by Neubauer haemocytometer. The qualifying ejaculates from five bulls were split into five aliquots and held for 15 min at 37°C in the water bath before dilution in five different experimental extenders.

Semen Processing

Semen aliquots were diluted in a single step at 37°C with one of the five experimental extenders at the rate 50×10^6 motile spermatozoa ml^{-1} approximately. Diluted semen was cooled to 4°C in 2 h at the rate of 0.275°C min^{-1} and was equilibrated for 4 h at 4°C. Semen was then filled in 0.5 ml French straws (IMV, France) with suction pump at 4°C in the cold cabinet unit (IMV, France) and kept on liquid nitrogen vapours for 10 min. Straws were then plunged and stored into liquid nitrogen (-196°C). After 24 h, semen straws were thawed in a water bath at 37°C for 30 s. For each extender, contents from three straws were pooled and incubated at 37°C in water bath for assessment of post-thaw semen quality at 0, 3 and 6 h after thawing.

Post-thaw sperm functional assays

Sperm quality assays comprised of sperm progressive motility, viability, plasma membrane and acrosomal integrity performed at 0, 3 and 6 h post-thawing.

 Progressive motility. A drop of thawed semen sample was placed on pre-warmed glass slide and covered with a cover slip. Progressive motility was assessed with a phase contrast microscope (X 200) at 37°C.

Viability. Sperm viability (live/dead percentage) was assessed using 0.4% Trypan blue stain. For this purpose, 5 μl semen sample was mixed with an equal amount of Trypan Blue stain to make a smear on a glass slide that was air dried. Air dried slides were examined under phase contrast microscope (X 1000, oil immersion). Spermatozoa stained blue were considered as dead with disrupted membranes; while unstained as alive with intact plasma membranes. A total of one hundred spermatozoa per experimental extender were counted.

Plasma membrane integrity Sperm plasma membrane integrity (PMI) of spermatozoa was assessed using hyposmotic swelling (HOS) technique. Solution used for HOS assay consisted of sodium citrate 0.73 g and fructose 1.35 g dissolved in 100 ml distilled water (osmotic pressure ~190 mOsmol Kg^{-1}). For the assessment of sperm plasma membrane integrity 50 μl of frozen-thawed semen sample was mixed with 500 μl of HOS solution and incubated for 30-40 min at 37°C after which a drop of the mixed sample was placed on glass slide and covered with a cover-slip

to examine under a phase contrast microscope (X 400). Swelling of spermatozoa characterized by a coiled tail was considered an intact sperm plasma membrane. One hundred spermatozoa per experimental extender were counted.

Acrosomal integrity. To assess the acrosomal integrity 100 µl semen sample was fixed in 500 µl of 1.0% formal citrate (2.9 g tri-sodium citrate dehydrate, 1 ml of 37% solution of formaldehyde, dissolved in 100 ml of distilled water). Normal acrosome was characterized by normal apical ridge (NAR). One hundred spermatozoa per experimental extender were counted with phase contrast microscope (X 1000) under oil immersion.

Statistical analysis

The data are presented as means and standard deviations (SD) of different parameters used to assess the post-thaw quality of the semen samples diluted in different semen extenders. Significance of relations was analyzed by analysis of variance; when appropriate, Post-hoc comparisons for treatment were performed using LSD. A level of 5% (P<0.05) was used to determine statistical significance (MSTAT-C Ver.1.42).

Results and discussion

The present study was aimed to investigate the effect of pre-freezing glutathione addition to the extender on the post-thaw quality of buffalo semen. A range of glutathione concentrations were used and the semen quality was assessed at 0, 3 and 6 h after thawing. The parameters used to assess the semen quality included: progressive motility, viability, plasma membrane integrity and percentage of sperms with intact acrosomes.

Progressive motility

The data on the effect of glutathione in semen extender on the post-thaw motility (%) of buffalo bull spermatozoa is presented in Figure 1. Supplementation of glutathione in semen extender significantly (P≤0.05) increased post-thaw progressive sperm motility examined at 0, 3, 6 hours after thawing in a dose- manner and the highest sperm motility was observed at 2.0 mM glutathione (56.7±2.9, 41.7±2.9, 28.3±2.9). Moreover, there was no beneficial effect of higher 3.0 mM glutathione concentration for progressive sperm motility of buffalo semen. Physico-chemical properties of a diluent can affect the pattern of sperm motility and in this study these properties might have been altered by the addition of glutathione in the semen extender before freezing. Results of the present study have shown that post-thaw sperm progressive motility was significantly increased in a dose-dependent manner with glutathione supplementation in the extenders upto 2.0 mM concentrations compared to the controls or higher (3.0 mM) GSH concentrations after 0, 3 and 6 hours incubation at 37°C. Our findings are in line with the results of such studies in bovine [Bilodeau *et al*. 2001] and caprine
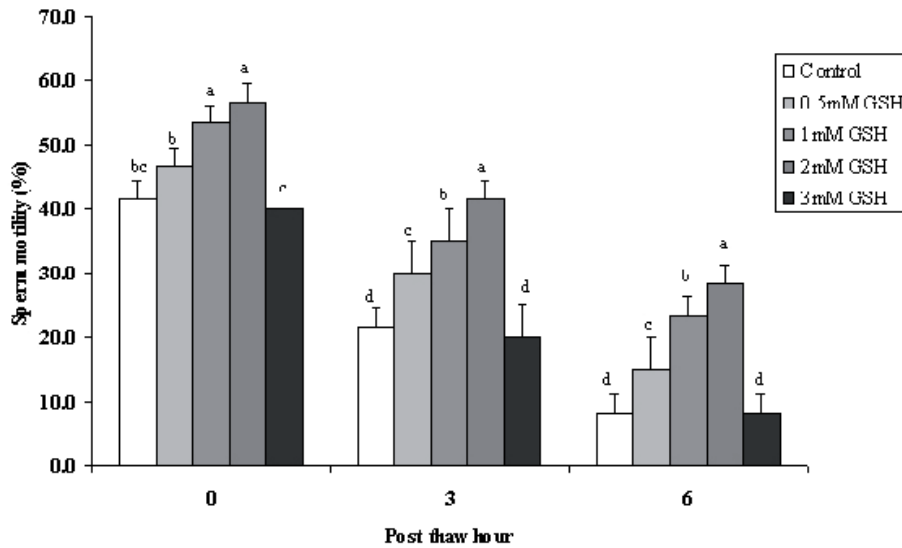
Fig. 1. Effect of glutathione (GSH) supplementation in the semen extender on the progressive motility of buffalo bull spermatozoa at 0, 3 and 6 h after thawing. Bars with different letters show significant (P≤0.05) differences at a given time.

semen [Sinha *et al*. 1996], in which increase in post thaw sperm motility has been reported after the fortification of extenders with GSH. This increase in sperm motility with glutathione supplementation might be due to a decrease in oxidative stress and ROS production that is associated with lipid peroxidation of the sperm plasma membrane causing poor sperm motility.

Viability

The data on the effect of glutathione in extender on the post-thaw viability of buffalo semen are presented in Figure 2. Addition of glutathione in semen extender increased (P≤0.05) percentage of viable spermatozoa in a dose-dependent manner evaluated at 0, 3, 6 hours after thawing and the maximum values for sperm viability were recorded at 2.0 mM glutathione (89.0±2.0, 75.3±2.5, 59.3±4.9). The higher dose of glutathione (3.0 mM) had no beneficial effect on post-thaw sperm viability of buffalo semen. The number of viable sperms per dose of frozen-thawed semen significantly affects fertility rates in the field. In the present study, post-thaw viable sperm number was significantly higher in the extender containing 2 mM glutathione followed by those containing 1.0 mM and 0.5 mM as compared to 3.0 mM or the controls at 0, 3 and 6 hours incubation at 37°C. Addition of GSH to the semen extender improved the number of viable sperms in post-thaw caprine semen [Sinha *et al*. 1996] while a decrease in sperm viability was associated with decreased glutathione
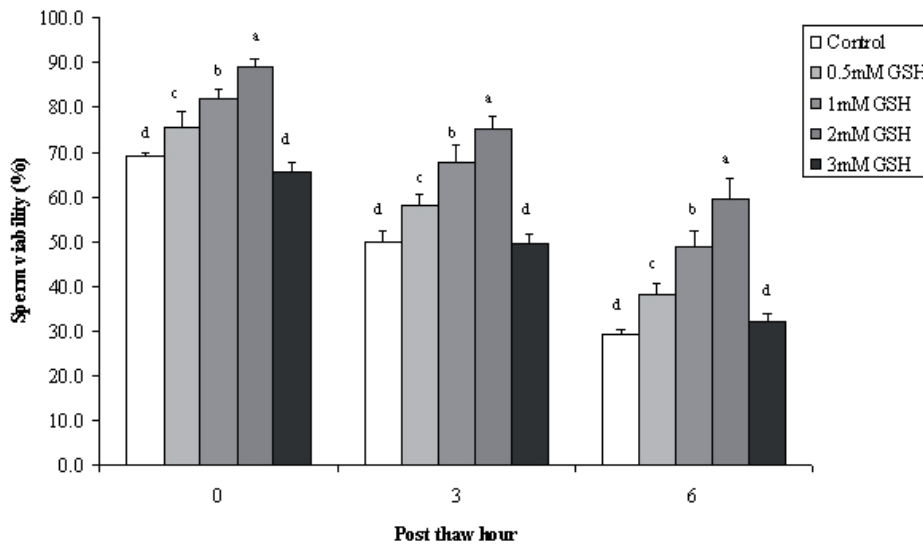
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Fig. 2. Effect of glutathione supplementation in the semen extender on the viability of buffalo bull spermatozoa at 0, 3 and 6 h after thawing. Bars with different letters indicate significant difference (P≤0.05) at a given time.

content of the porcine semen [Gadea *et al.* 2004]. Moreover, an improvement in sperm viability has been reported to be highly correlated with glutathione content of the bull spermatozoa [Stradaioli *et al.* 2007]. These findings support our results of an improvement in buffalo sperm viability with glutathione supplementation. It is to believe that glutathione protects the sperm viability through scavenging the lipid peroxides as oxidative agents can initiate a chain reaction that destroys the sperm cell membrane resulting into cellular aging.

Plasma membrane integrity

The data on the effect of glutathione in freezing extender on the percentage of post-thaw sperm plasma membrane integrity of buffalo spermatozoa is presented in Figure 3. Fortification of semen extender with glutathione significantly (P≤0.05) increased the sperm plasma membrane integrity evaluated at 0, 3 and 6 h after thawing in a dose-manner and the highest values of sperm plasma membrane integrity were observed at 2.0 mM glutathione (88.7±0.6, 70.3±2.1, 58.7±6.7, respectively). Glutathione at the dose of 3.0 mM did not show any improvement in the percentage of spermatozoa with intact plasmalemma. The sperm plasma membrane plays a critical role in controlling sperm fertilizing ability; not only does it directly mediate the contact interactions between the spermatozoa and the oocyte itself, but it also acts as the receiver of the environmental signals that induce the positive response the spermatozoa must make in order to achieve fertilization. Structural and functional integrity of the sperm plasma membrane is essential for fertilization process. The evaluation of plasma membrane
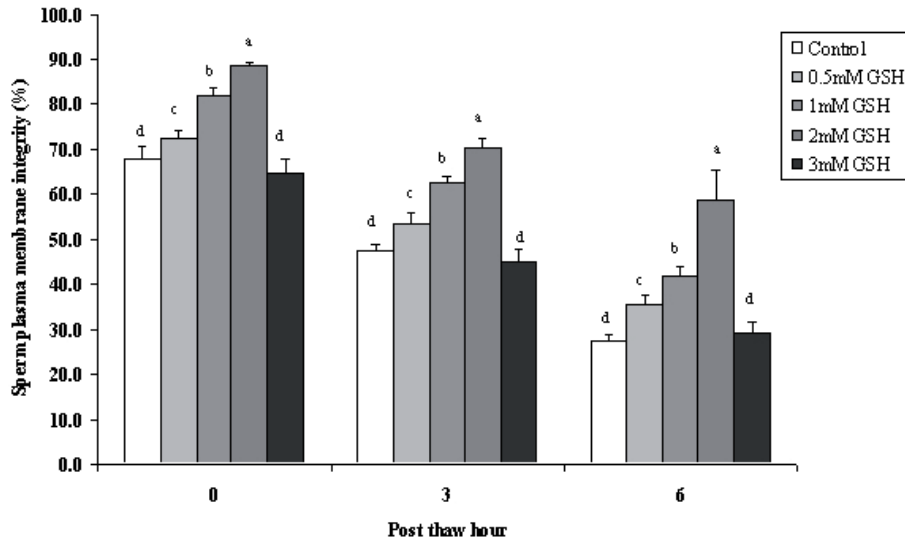
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Fig. 3. Effect of glutathione (GSH) supplementation in the semen extender on the plasma membrane integrity of buffalo bull spermatozoa at 0, 3 and 6 h after thawing. Bars with different letters indicate significant (P≤0.05) differences at a given time.

integrity is of particular importance due to its involvement in metabolic exchange with the surrounding medium. Furthermore, the processes of capacitation, acrosome reaction and the oocyte penetration require a biochemically active plasma membrane. In the present study, sperm numbers with intact plasma membrane at 0, 3, and 6 h after thawing at 37 °C were higher in the extenders containing upto 2 mM glutathione as compared to 3.0 mM GSH or the control. Recently, GSH supplementation of the extender was reported to improve the quality of the preserved ovine spermatozoa [Bucak and Tekin 2007]. Nevertheless, it is important to mention that the data on improvement in the post-thaw plasma membrane integrity are strongly supported by the observed improvement in the sperm progressive motility with GSH supplementation of the extenders. In the sperm cell, oxidative stress accelerates the over-production of ROS, resulting in lipid peroxidation of the cell membrane. Moreover, oxidative metabolites injure the spermatozoa chemically and physically using different pathways. Since glutathione plays an important role in scavenging reactive oxygen intermediates and other radicals with the help of glutathione reductase [Meister and Andersson 1983], it is a possibility that glutathione might protect the spermatozoa from membrane damage by inhibiting the lipid peroxidation process [Sinha *et al*. 1996].

Sperm normal apical ridge

The data on the effect of glutathione in extender on the post-thaw normal apical ridge (%) of buffalo spermatozoa are presented in Figure 4. Supplementation of semen extender with glutathione significantly (P≤0.05) increased normal acrosomes
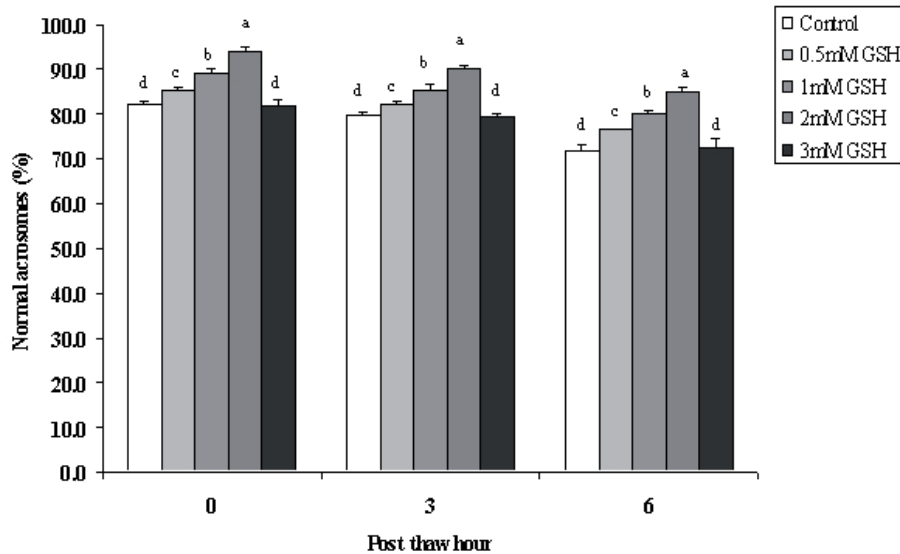
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Fig. 4. Effect of glutathione (GSH) supplementation in the semen extender on the acrosomal integrity of buffalo bull spermatozoa at 0, 3 and 6 h after thawing. Bars with different letters indicate significant (P≤0.05) differences at a given time.

examined at 0, 3, and 6 h after thawing in a dose-manner and the highest values of normal apical ridge were observed at 2.0 mM glutathione (94.0±1.0, 90.0±1.0, 85.0±1.0, respectively). Addition of glutathione at a dose of 3.0 mM in semen extender showed no beneficial effect on the normal apical ridge. The acrosome is a large golgi/endoplasmic reticulum-derived acidic secretory organelle. It is filled with hydrolytic enzymes that are organized in a kind of enzyme matrix and most enzymes are heavily glycosylated. The presence of normal acrosome on a spermatozoon is essential for the acrosomal reaction that is required at the proper time to facilitate fertilization [Baily *et al*. 2000]. Acrosomal integrity assessment can be an effective tool to predict the fertilizing ability of buffalo bull spermatozoa. In the present study, percentage of spermatozoa with intact acrosomes at 0, 3, and 6 h post-thawing at 37°C was significantly higher in the extenders containing upto 2 mM glutathione compared to 3.0 mM or the control. These results confirm the earlier report on caprine semen [Sinha *et al*. 1996] in which glutathione supplementation has been found to decrease the post-thaw acrosomal damage and release of aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase enzymes from the sperm in the surrounding medium. Moreover, in a recent study by Munsi *et al*. 2007], an addition of GSH in bovine semen has been reported to result in a higher number of acrosome intact spermatozoa.

In conclusion, glutathione supplementation (up to 2.0 mM) of the extender improves post-thaw quality of the buffalo bull spermatozoa. However, this can only be recommended for artificial insemination programmes in the buffalo if supported by an improvement in the fertility results.

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